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The genetic status of two insular populations of the endemic spiny rat *Tokudaia osimensis* (Rodentia, Muridae) of the Ryukyu Islands, Japan

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Abstract. We examined the geographic variation of *Tokudaia osimensis* through the analysis of mitochondrial cytochrome *b* (cyt *b*) gene sequences and the restriction fragment length polymorphism (RFLP) in the nuclear ribosomal RNA gene (rDNA), using samples collected from Tokuno-shima and Amami-oshima in the Ryukyu Islands. The two populations show intrinsic karyological variation (Tokuno-shima, 2n=45; Amami-oshima, 2n=25). Sequences of the cyt *b* gene differed considerably between the two island populations. The extent of the sequence divergence among 1,140 bp of the gene was calculated to be 0.088 using the Kimura two parameter method, and was comparable to those between related species of rodents such as within genus *Mus* or *Rattus*. The extent of the differentiation in the rDNA-RFLP was also high. Three out of 22 restriction site variants were found to be fixed in the nuclear rDNA arrays of hundreds of copies in either one of the two island populations. These intensive inter-populational differences indicate that the two island populations may have been isolated for a considerable period of evolutionary time, probably several millions of years, despite there having been several opportunities for renewed genetic contact during the Pleistocene ice ages. Our data strongly suggest that the current taxonomic status of the populations of the two islands, Amami-oshima and Tokuno-shima, which regards them conspecific, should be reviewed.

Key words: geographic variation, cytochrome *b* gene, nuclear rDNA-RFLP, Ryukyu Islands, *Tokudaia osimensis*.

The Nansei Shoto or Ryukyu Islands, Japan's southernmost islands, harbor a unique fauna and flora, and the central region, consisting of the three islands of Amami-oshima, Tokuno-shima and Okinawa and adjacent islets, is especially rich and a center of endemism. Three genera of the following mammals are endemic to this small area of islands: the Ryukyu spiny rat *Tokudaia osimensis*; the Ryukyu long-haired rat *Diplothrix legata*; and the Amami

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rabbit *Pentalagus furnessi* (Corbet and Hill 1991; Musser and Carleton 1993; Hoffmann 1993; Abe 1994; Kaneko and Murakami 1996). Because of their uniqueness and zoological importance, all three are protected as natural monuments by the Japanese government and also regarded as endangered species (IUCN 1996; Kawamichi 1997). These three species are also considered to be symbolic of the significant biodiversity of the Central Ryukyus, and for the conservation of the fauna of the islands. Moreover, their distribution and status provides invaluable information towards an understanding of the historical episodes of the Ryukyu fauna as well as contributing to an understanding of general evolutionary issues.

Tokudaia osimensis, in particular, raises various interesting scientific issues. The species shows intrinsic karyological features in its autosomes and sex chromosomes, with the diploid numbers of both females and males being 44 in Okinawa, 45 in Tokuno-shima, and 25 in Amami-oshima (Honda et al. 1977, 1978; Tsuchiya et al. 1989). Since the Y chromosome in the Tokuno-shima and Amami-oshima island populations disappears (Honda et al. 1977, 1978), and the animals from Amami-oshima are shown to lack the Sry gene (Soullier et al. 1998), an unusual sex-determining system must have been evoked in these populations (Honda et al. 1977; Tsuchiya et al. 1989; Soullier et al. 1998; Xiao et al. 1998). However, neither the evolutionary process leading to these differences, nor the biological implications of the differences have been elucidated.

The evolutionary history of the populations of *T. osimensis* and the origin of this lineage have long been debated (for review see Kawamura 1989). A recent molecular phylogenetic study (Suzuki et al. 1999b) has revealed that *T. osimensis*' lineage is distinct from the other members of the subfamily Murinae examined so far, including *Apodemus*, which had been considered a likely candidate for the sister lineage of *Tokudaia* based on molar morphology (Kawamura 1989). An assessment of the genetic diversity within this species is inevitably needed for back ground information to elucidate the above biological problems, and to resolve the unsettled taxonomic status of the three island populations of *T. osimensis*. Although recently each of the three populations is presumed to be a distinct species (Honda et al. 1977; Tsuchiya et al. 1989; Musser and Carleton 1993), there has only been limited research on this species with such complicated genetic property using molecular markers (Tsuchiya et al. 1989).

In a preliminary study using a limited number of restriction enzymes we found substantial genetic differences between populations of *T. osimensis* from Tokuno-shima and Amami-oshima, based on restriction fragment length polymorphisms (RFLP) of mitochondrial DNA (mtDNA) and nuclear ribosomal RNA genes (rDNA) (Tsuchiya et al. 1989). This study was conducted therefore to improve our knowledge of the molecular phylogeny of the Tokuno-shima and Amami-oshima island populations, by examining a whole sequence for the mitochondrial cytochrome *b* (cyt *b*) gene and the rDNA-RFLP with more additional restriction enzymes.

Materials and methods

Animals

We have tentatively followed Abe's (1994) classification and accepted that *Tokudaia* consists of just one species (*T. osimensis*) with three island populations. Five individuals of *T. osimensis* were examined, two from Tokuno-shima and three from Amami-oshima. With

the exception of one sample from Amami-oshima, the samples were the same as those previously used by Tsuchiya et al. (1989). The new sample (sample no. HS1142) from Amami-oshima was collected at Tatsugo, 28 February 1996 through a wildlife survey conducted by Japan Wildlife Research Center (Environment Agency of Japan 1995).

Sequencing and phylogenetic analysis

Nuclear DNA extraction, Southern blot analysis and the construction of restriction maps for the rDNA repeating unit type (repetype), were all carried out following Suzuki et al's (1994a) methodology. The cyt *b* region was analyzed using nested polymerase chain reactions and a direct sequencing method as described previously by Suzuki et al. (1997, 1999b).

In order to estimate the sequence divergence from restriction site variation among rDNA repetypes, we compared the arrangement of the restriction sites between the pairs of repetypes and then counted the common and divergent sites (Suzuki et al. 1994a). To do this, we used Gotoh et al's (1979) method, in which backward mutations and parallel mutations are taken into account, to produce a matrix of sequence divergence among all possible combinations of repetypes.

To estimate the sequence divergences from sequences of the cyt *b* gene, we used the two parameter method (Kimura 1980) and MEGA (Kumar et al. 1993).

Results

Cyt b sequences

Fragments of the cyt *b* gene, from each of the five specimens, consisting of 402 bp were determined, and it was found that each island population had unique sequences. We then sequenced the entire gene region of the cyt *b* gene of one individual from Tokuno-shima (the nucleotide sequence can be reached in the DDBJ, EMBL and GenBank with following accession number: AB029429) and compared it with that of the previously described sample from Amami-oshima (Suzuki et al. 1999b), calculating sequence divergences (see Kimura 1980) taking into consideration complete substitution (*d*), and only transversional substitution (*dv*; see Table 1). The extent of transversional substitution amounted to 0.026, which is comparable to that between species of *Rattus-Diplothrix* and *Mus* (*dv*=0.014–0.016; Table 1). The extent of complete substitution (*d*=0.088) was also extremely high when compared with other cases of intraspecific sequence divergences within mammalian species (Avise et al. 1998; Johns and Avise 1998; Table 1 for the case of *Glirulus japonicus*) and rather comparable to those among congeneric mammalian species (Johns and Avise 1998). Such high degrees of divergence in the cyt *b* sequences were congruent with our previous study with the mtDNA-RFLP (Tsuchiya et al. 1989).

rDNA-RFLP

We carried out Southern blot analysis with the two island samples using 12 restriction enzymes. Among the enzymes examined the *Kpn*I bands, with both the 18S and 28S probes, remained at higher molecular weight position without any indication of digestion with this enzyme in samples from both islands. Thus, we considered there to be no *Kpn*I site located in the spacer region in these populations. Restriction maps were constructed taking into consideration the banding patterns (Fig. 1). Interestingly, the Amami-oshima sample's

Table 1. Comparison of sequence divergences between related species and among geographic populations in small mammals. Sequence divergences in the cytochrome *b* gene (1,140 bp) were calculated using Kimura's (1980) two parameter method considering all substitutions at all codon positions (*d*) and transversions at all codon positions (*dv*).

Taxa compared	Substitution considered	
	d	*dv*
Between geographic populations		
1. *Tokudaia osimensis*		
'Amami-oshima' vs 'Tokuno-shima'	0.088	0.026
2. *Mus musculus**		
M. m. domesticus vs *M. m. musculus*	0.024	0.004
3. *Glirulus japonicus***		
'Wakayama' vs 'Yamanashi'	0.075	0.012
Between species within *Mus* and *Rattus**		
4. *M. musculus* vs *M. spretus*	0.091	0.014
5. *R. rattus* vs *Diplothrix legata*	0.102	0.016
Between genera *Mus* and *Rattus**		
6. *M. musculus* vs *R. norvegicus*	0.186	0.082

* Suzuki et al. (1999b). The genus *Diplothrix* is a member of a *Rattus* group in the molecular phylogenetic view.

** Suzuki et al. (1997; unpublished data)

repeating type was heterogeneous within a genome as depicted in the *Hin*dIII and *Xba*I sites upstream of the 18S rRNA gene and *Eco*RI, and *Dra*I sites downstream of the 28S rRNA gene (Fig. 1). In contrast, the banding patterns of the Tokuno-shima specimens were monotypic within a genome at each restriction site. These phenomena can be explained either by there being a large population on Amami-oshima or by some prevention of the homogenization process (including DNA recombination) within a genome in the Amami-oshima population. The former postulates that the banding patterns of individuals from a large population size tend to show polymorphic state rather than those from a small population size (Suzuki et al. 1994b). Although there is no substantial data on population size of this species, the area of Amami-oshima is about three times as large as that of Tokuno-shima. In the latter case, if rDNA clusters coexist onto terminal and interstitial regions of chromosomes, recombination between non-homologous chromosomes would be unfavorable since it may cause abnormal chromosomal changes with serious damage to the cell. We just presume a possibility that a rDNA cluster(s), which often locate distal portions of chromosomes accompanied by heterochromatic regions but not euchromatic one, as in the cases of *Mus* and *Rattus* (Babu and Verma 1985), incorporated into inside chromosomes by chromosomal rearrangement in the Amami-oshima population (2n=25).

The difference between the geographic populations became more conspicuous during examination of the rDNA-RFLP. According to Gotoh et al's (1979) method, inter-populational sequence divergence was calculated to be approximately 2.3%. This extent was considered likely to be as high as between distantly related local populations such as of the Japanese dormouse *Glirulus japonicus* (2.9–3.3%, Suzuki et al. 1997), and between closely related species of red-backed voles (genera *Clethrionomys* and *Eothenomys*) in Japan (1.9–2.3%, Wakana et al. 1996; Suzuki et al. 1999a). Since each variant spreads over the arrays

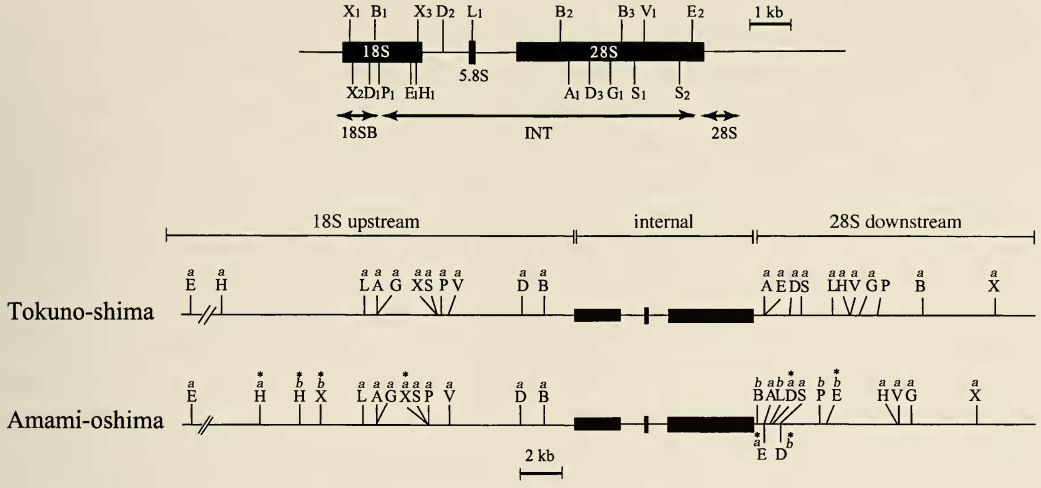

Fig. 1. Restriction maps of the rDNA repeating units of Tokuno-shima and Amami-oshima populations of *Tokudaia osimensis*. Each rDNA repeating unit is composed of three rRNA genes (28S, 5.8S, and 18S rRNA) which are separated from each other by spacers. With respect to the restriction sites on the flanking spacers, only those nearest to the distal end of the genes for 18S or 28S rRNA are shown. The upper diagram shows the conserved restriction sites in the coding and the internal spacer regions of the genes for 18S and 28S rRNA, which are not represented in the lower maps. The positions of the probes are also shown by arrows. Letters with superscripts represent specific types of restriction sites identified after comparison with the restriction maps. Types of Tokuno-shima are treated as *a*. Asterisks indicate polymorphic sites within individuals. A=*Aat*I; B=*Bam*HI; D=*Dra*I; E=*Eco*RI; G=*Bgl*II; H=*Hin*dIII; K=*Kpn*I; L=*Bcl*I; P=*Pst*I; S=*Sac*I; V=*Pvu*II; and X=*Xba*I.

of rDNA within a population through certain homogenization mechanisms (Coen et al. 1982), the presence of several distinct variants between the two islands clearly indicates that the two populations have been isolated for a considerable period of time. The amount of the sequence divergence, 2.3%, corresponds to a divergence time of 1.2–2.3 million years, if we assume that the divergence rate is 1–2% per million years (Suzuki et al. 1994a, 1999a).

Discussion

During this study we detected considerable differences in the cyt *b* sequences and the rDNA-RFLP between populations of *T. osimensis* from the islands of Tokuno-shima and Amami-oshima, as previously predicted by karyological analysis (Honda et al. 1977, 1978; Tsuchiya et al. 1989) and our preliminary molecular analysis (Tsuchiya et al. 1989). The difference between the populations, and the extent of the divergence in the two molecular markers, has greatly improved our knowledge of the evolutionary processes of these island populations. Our data will be helpful in assessing the evolutionary history and in reconsideration for taxonomic status of this species.

The extent of the inter-populational variation in the cyt *b* sequences between the two island populations was comparable to that between *Rattus* species and *Diplothrix legata* (Table 1). These results suggest that certain kinds of populational differentiation began a very long time ago. Differentiation of genes under the ordinal inherited mode, however, does not always reflect populational differentiation. Furthermore, in the case of mtDNA,

the differentiation patterns do not reflect the movement of males. For example, the differentiation patterns of mtDNA in geographically separate populations of the Japanese dormouse *Glirulus japonicus*, and Smithii's red-backed vole *Eothenomys smithii*, are not congruent with those of nuclear genes and morphological types (Suzuki et al. 1997, 1999a; Iwasa et al. unpublished). In contrast, data sets of the nuclear rDNA, a member of multigene families, would provide more useful information on the genetic status of given populations. The rDNA consists of several hundred copies within a genome, and a given variant extends to all of the units by certain homogenization mechanisms, and to all of the genomes of the same population as a result of mating (Dover 1980; Ohta 1980). In the case of *T. osimensis*, of the 22 restriction sites examined, three sites were completely differentiated, and four more were under differentiation between the rDNA repeating units of the two islands (Fig. 1). This data implies that the two island populations of *T. osimensis* have been reproductively isolated for some million years, despite there having been many chances to exchange genetic elements during the Pleistocene ice ages when falling sea levels led to land bridges existing between the islands (Kimura 1996). We could conclude, therefore, that these two island populations are already genetically differentiated to such an extent that there is little or no probability of future genetic contact. Consequently, the spiny rats from the two islands of Amami-oshima and Tokuno-shima may be better regarded as two independent species.

This assumption is congruent with the observed karyological differentiation between the island populations in which the diploid numbers are $2n=45$ (Tokuno-shima) and $2n=25$ (Amami-oshima). From the karyological perspective (Honda et al. 1977; Tsuchiya et al. 1989), such populations would not be expected to produce fertile progeny, that is they have been reproductively isolated through certain post-mating isolation mechanism. Such information clearly brings into question the current taxonomic status and suggests that the status of *T. osimensis* as a monotypic species should be reconsidered (Honda et al. 1977; Tsuchiya et al. 1989; Musser and Carleton 1993; Kaneko and Murakami 1996).

Interestingly, the extent of the cyt *b* divergence between these two island populations is somewhat similar to the level of distinctness of *D. legata* (Suzuki et al. 1999b; Table 1). This may imply that some geological event was attributable to both the geographical divergence of *T. osimensis* and to the migration and colonization of *D. legata* in the Okinawa Islands. The most simple explanation for such differentiation is that it was triggered by the disappearance of land bridges that once connected the islands of the region. Our rough time estimation predicts that divergence occurred 3.8–4.9 million years ago (Mya), taking into account the extent of transversional substitution (Table 1) and using the "standard" time estimation of the rat-mouse split as 12–14 Mya (though others have estimated the rat-mouse split to be more ancient (20–29 Mya, O'hUigin and Lee 1992; 40 Mya, Kumar and Hedges 1998)). If the time frame is estimated on the basis of a total-substitution rate of 2% per million years (Brown et al. 1979), then divergence is estimated to be 4.4 Mya. Both of these estimates related well to the geological view that the Ryukyu Islands were once connected to the Asian continent but became disconnected during the beginning of the Pleistocene, around 1.7 Mya (Kimura 1996). It may be postulated that such geological changes affected the differentiation of *D. legata* from other continental sister *Rattus* lineages, and simultaneously triggered the geographic divergence of the mtDNA haplotypes in *T. osimensis*.

In order to fully understand the various important issues related to the status of *T.*

osimensis, comparable data for *T. osimensis* from the Okinawa, is required. Karyologically, the Okinawan population represents the normal type, and may represent the ancestral situation of the unusual karyotypes. Studies of the Okinawan population are essential for the investigation of other issues such as the geographical differentiation of genes, and the taxonomic reconsideration of the island populations. Despite the scientific importance, all three populations of this taxon, especially in Okinawa, are thought to have already decreased to a point where those are seriously endangered possibly due to habitat destruction, predation by and competition with introduced species such as the Javan mongoose *Herpestes javanicus*, the feral cat *Felis catus* and the feral dog *Canis familiaris*, and the black rat *Rattus rattus*. The Environment Agency (1995) listed the Okinawan population as critically endangered and the populations of Amami-oshima and Tokuno-shima as endangered in the national Red List. Given the current status of *T. osimensis* in the wild in Okinawa, and given the remarkable biological importance of this taxon, effective conservation efforts are required, and these may include a research project to promote their reproduction in captivity while the issue of alien predators is dealt with.

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